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**Latent Cytomegalovirus infection amplifies CD8 T-lymphocyte mobilisation and egress in response to exercise**

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## Abstract

Exercise induces a mobilisation of CD8<sup>+</sup> T lymphocytes (CD8TL) into peripheral blood. This response is largely confined to effector-memory CD8TLs: antigen experienced cells which have a strong tissue-homing and effector potential. This study investigated whether T cell memory phenotype also accounts for the CD8TL egress from peripheral blood following exercise. As latent Cytomegalovirus (CMV) infection is associated with a robust expansion in the number and proportion of effector-memory CD8TLs, we also investigated if CMV serostatus was a determinant of the CD8TL responses to exercise.

Fourteen males (Mean age 35, SD  $\pm$  14 yrs), half of whom were CMV seropositive (CMV<sup>+</sup>), ran on a treadmill for 60 minutes at 80%  $\dot{V}O_2$  max. Blood was collected at baseline (Pre), during the final minute of exercise (Ex60), and 15 (Post15) and 60 (Post60) minutes thereafter. CD8TL memory subsets were characterised by flow cytometry, using the cell-surface markers CD45RA, CD27, and CD28.

The results confirmed that CD8TLs with an effector-memory phenotype (CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+/+</sup>) exhibited the largest increase during exercise (+200% to +250%), and further showed that these cells exhibited the largest egress from blood 60 minutes post-exercise (down to 40% of baseline values). Strikingly, the mobilisation and subsequent egress of total CD8TLs was nearly twice as large in CMV<sup>+</sup> individuals. This effect appeared specific to CD8TLs, and was not seen for CD4<sup>+</sup> T lymphocytes or total lymphocytes. This effect of CMV was largely driven by higher numbers of exercise-responsive effector-memory CD8TLs in the CMV<sup>+</sup> participants.

This is the first study to demonstrate that infection history is a determinant of immune responses to exercise.

50 **Key words:** Cytomegalovirus; CD8<sup>+</sup> T lymphocytes; memory lymphocytes; lymphocytosis;  
51 lymphocytopenia; immune surveillance; migration; exercise; stress; human.

## 1. Introduction

All mammals, including humans, have a remarkable capacity to acutely change the cellular composition of peripheral blood in response to psychological and physiological stressors (Dhabhar, 2000). For example, strenuous exercise causes a near-immediate mobilisation of lymphocytes into the blood, a process referred to as lymphocytosis (Gleeson and Bishop, 2005; McCarthy and Dale, 1988). This phenomenon is particularly marked for lymphocytes with a cytotoxic potential, such as Natural Killer cells,  $\gamma\delta$  T lymphocytes, and CD8<sup>+</sup> T lymphocytes (CD8TLs) (Anane et al., 2009). These subsets exhibit a high adrenergic receptor density and sensitivity, which in turn, regulates their detachment from vascular endothelium and release into the blood (Anane et al., 2009; Benschop et al., 1994; Dimitrov et al., 2010). Indeed, there is now conclusive evidence that stress and exercise-induced lymphocytosis is under the control of the sympathetic nervous system and the concomitant release of catecholamines (Benschop et al., 1994; Dimitrov et al., 2010; Elenkov et al., 2000).

One to two hours after high-intensity exercise, the lymphocytosis is followed by a lymphocytopenia whereby the numbers of circulating lymphocytes fall below baseline level (Gleeson and Bishop, 2005; McCarthy and Dale, 1988). Experimental studies in rodents suggest that this lymphocytopenia reflects extravasation of lymphocytes to selected peripheral tissues, such as the lungs, presumably part of enhanced immunosurveillance in front-line tissues (Krüger et al., 2008; Krüger and Mooren, 2007). Consistent with this model, recent studies in humans have shown that exercise preferentially mobilises a subset of memory CD8TLs that have a strong tissue migrating potential and the capacity to induce rapid effector responses (e.g., target killing) (Campbell et al., 2009; Simpson et al., 2008; Simpson et al., 2007a). It might therefore be

74 predicted that these tissue-migrating memory cells would preferentially egress from peripheral  
75 blood post-exercise, but there is currently no data to support this contention.

76 Subsets of CD8TLs have been identified on the basis of the combined expression of cell  
77 surface markers such as CD45RA, CD27, and CD28 (Appay et al., 2002; Appay et al., 2008;  
78 Hamann et al., 1997; Romero et al., 2007) (see Table 1). In our study we used two phenotypic  
79 profiles to define effector memory subpopulations; comparing CD45RA and CD27 expression,  
80 or CD27 and CD28 expression (see Table 1). CD45RA is one of the two isoforms of the  
81 leukocyte surface protein CD45R. This isoform is present on naïve T cells and replaced by the  
82 isoform CD45RO after antigenic recognition. However, CD45RA is re-expressed on a subset of  
83 so-called ‘revertant’ memory cells, and this CD45RA<sup>+</sup>CD27<sup>-</sup> phenotype is known to exhibit  
84 strong effector potential (such as the ability for rapid target killing, inflammatory cytokine  
85 production, and tissue migration, see Table 1). CD27 and CD28 are co-stimulatory and survival  
86 molecules that are co-expressed on naïve and early memory populations. However, during  
87 differentiation (i.e., a process characterised by the accumulation of effector functions), these  
88 markers are gradually lost and a CD27<sup>-</sup>CD28<sup>-</sup> phenotype defines a population of late  
89 differentiated effector memory cells. CD45RA<sup>+</sup>CD27<sup>-</sup> cells are themselves largely CD28<sup>-</sup> and  
90 there is therefore considerable overlap between these phenotypic profiles (Appay et al., 2008;  
91 Hamann et al., 1997; van Lier et al., 2003)

92 Individual differences in the proportions and numbers of the various CD8TL memory  
93 subsets are to a considerable extent determined by infection history (Chidrawar et al., 2009;  
94 Khan et al., 2002). For example, infection with Cytomegalovirus (CMV) causes a robust  
95 accumulation of late differentiated effector-memory CD8TLs, which are characterised by loss of  
96 CD27 and CD28 expression, as well as frequent ‘reversion’ to expression of the naïve CD45RA

isoform (Appay et al., 2002; van Lier et al., 2003). CMV is an endemic herpes virus which infects approximately 60% of western populations (Lubeck et al., 2010; Staras et al., 2006). In healthy individuals infection with CMV is usually asymptomatic and the virus remains latent in the body, but is believed to undergo intermittent reactivation (Stowe et al., 2007; Zanghellini et al., 1999). In view of research showing a selective mobilisation of effector memory CD8TLs with exercise (Campbell et al., 2009), we investigated if CMV seropositive individuals demonstrated an exaggerated CD8TL exercise-response as a consequence of the accumulation of late-differentiated/effector-memory CD8TLs.

The aim of the current study was therefore to determine the pattern of egress within discrete CD8TL memory subsets following recovery from exercise and to compare this to the profile of mobilisation during exercise. Secondly, we aimed to determine if infection history is a determinant of this immune cell redistribution with a specific investigation of how CMV serostatus influences the magnitude and kinetics of CD8TL mobilisation.

## 2. Methods

### 2.1. Participants

Participants were fourteen healthy non-smoking men (mean age = 35, SD  $\pm$  14; BMI;  $24 \pm 3 \text{ kg}\cdot\text{m}^{-2}$ ), who were accustomed to vigorous endurance exercise and had a cardio-respiratory fitness ( $\dot{V}\text{O}_2 \text{ max}$ ) within the 90<sup>th</sup> percentile for their age (Whaley et al., 2006). Participants provided informed consent, and the study was approved by the School of Sport and Exercise Sciences research ethics committee, of the University of Birmingham.

### 2.2. Procedures

Participants visited the laboratory to undergo two graded exercise tests on a treadmill. The first exercise test measured  $\dot{V}\text{O}_2 \text{ max}$  by running to volitional exhaustion, to enable the intensity of the exercise trial to be prescribed relative to the fitness of each participant (i.e., 80% of  $\dot{V}\text{O}_2 \text{ max}$ ). A second test assessed the relationship between oxygen consumption ( $\dot{V}\text{O}_2$ ) and four sub-maximal running speeds (range  $8.2 - 11.6 \text{ km}\cdot\text{h}^{-1}$ ) to calculate the speed to elicit 80% of  $\dot{V}\text{O}_2 \text{ max}$ , using linear regression. Breath-by-breath measurements were recorded every 5 seconds throughout both tests (Oxycon Pro, Jaeger, Germany), with heart rate monitored (RS200, Polar, Finland) and ratings of perceived exertion (RPE) (Borg, 1973) recorded during the final minute of each stage. In addition, participants had leisure time physical activity assessed using the international physical activity questionnaire (IPAQ) (Craig et al., 2003). IPAQ data was expressed as MET-minutes per week, where 1 MET (metabolic equivalent) is equal to resting metabolic rate (Ainsworth et al., 1993).

### 2.3. Exercise trial



One week after the preliminary tests, participants visited the laboratory for an exercise trial. Participants were instructed to refrain from exercising and drinking alcohol or caffeine during the day prior to the trial. After an overnight fast, participants reported to the laboratory between 06:00-07:00. Following a 15 minute rest, a baseline blood sample (Pre) was collected from forearm vein by an indwelling catheter, which was kept patent by flushing regularly with saline.

The baseline sample was followed by the exercise trial, which consisted of treadmill running at a speed to elicit 80% of  $\dot{V}O_2$  max for 60 minutes. Breath-by-breath measurements, heart rate and RPE were recorded for periods of 5 minutes at regular intervals. Blood samples were collected during the final minute of exercise (Ex60), and again 15 minutes (Post15) and 60 minutes (Post60) post-exercise. To address possible confounding of exercise results by diurnal variation in lymphocyte counts, seven participants also completed a control trial which involved sitting in the same room for 2 hours, with all measures collected in an identical manner to the exercise trial. The order of the two trials was counterbalanced.

#### *2.4. Flow cytometry*

Blood was collected in ethylene-diamine-tetra-acetic acid (EDTA) vacutainer tubes (Becton-Dickinson, Oxford, UK) and samples were prepared within 3 hours of collection. Briefly, whole blood was incubated with the following monoclonal antibodies for 20 minutes at room temperature; CD45RA FITC, CD27 PE (PharMingen, San Diego, USA), CD3 PerCP, CD4 APC, CD8 APC-cy7 (BD Biosciences, San Jose, USA) and CD28 PE-cy7 (eBioscience, Hatfield, UK). Erythrocytes were lysed with FACS lysing solution (BD Biosciences, San Jose, USA) according to manufacturer instructions, and then centrifuged at  $250 \times g$  for 7 minutes.

Cells were re-suspended in 2% paraformaldehyde phosphate-buffered saline solution and stored in the dark at 4°C. Cells were read within 24 h on a six colour flow cytometer (BD FACS CANTO II, BD Biosciences), collecting 25,000 gated lymphocytes. The flow cytometer was regularly calibrated using Calibrite beads (BD Biosciences, San Jose, USA) and compensation adjustments were made prior to each run using single labelled antibody tubes. Data were analysed using FlowJo7 (Tree Star, Inc., Ashland, OR), and lymphocytes enumerated using a Coulter ACT<sup>diff</sup> haematology analyser (Beckman-Coulter, High Wycombe, UK).

The lymphocyte population was gated on the forward *versus* side-scatter and further identified by CD8 expression in combination with CD3 (Campbell et al., 2008). CD8TL expression of CD45RA and CD27 was examined to identify naïve, central memory, effector memory, and CD45RA<sup>+</sup> effector memory sub-populations as described elsewhere (Campbell et al., 2009; Hamann et al., 1997; Romero et al., 2007). CD8TLs were also examined for CD27 and CD28 expression to identify early, intermediate and late sub-populations, as described by Appay et al. (2002).

## 2.5. CMV serostatus

Plasma from baseline blood samples was assayed for IgG antibodies to CMV using a commercially available enzyme-linked immunosorbent assay (ELISA) (Biocheck, Inc. CA, USA) according to manufacturer instructions.

## 2.6. Statistical analyses

Data were inspected for normal distribution using the Kolmogorov Smirnov test. Non-normally distributed data were transformed logarithmically. Responses to exercise were

181 examined using repeated-measures Analyses of Variance (ANOVAs), with age and baseline cell  
182 count included as covariates where appropriate (Jennings and Stine, 2007). Differences between  
183 individual time points were examined using post-hoc paired samples *t*-tests. To confirm our  
184 parametric analyses, equivalent non-parametric tests were used. Statistical significance was  
185 accepted at the  $p < .05$  level. Data are presented as means  $\pm$  standard error of the mean (SEM)  
186 unless otherwise stated. Data were analysed using SPSS statistical package version 18.0 for  
187 Windows (SPSS Inc, USA).

### 3. Results

All participants completed the exercise trial successfully. Table 2 compares  $\dot{V}O_2$ , energy expenditure, heart rate, and perceived exertion during treadmill running with seated rest during the control trial

#### 3.1. Leukocyte subsets

Table 3 shows the summary data for leukocyte subsets. Exercise was associated with an acute leukocytosis, whereby total leukocyte count increased by +85% during exercise and remained elevated +22% above baseline values at 60 minutes post-exercise. The leukocytosis during exercise was mainly driven by a mobilisation of lymphocytes (+113%) and granulocytes (+66%), while 60 minutes after the exercise bout, lymphocyte numbers fell below baseline levels (−25%), accompanied by a secondary mobilisation of granulocytes (+67%) (See Table 3). The biphasic lymphocyte response was replicated in both CD4+ T lymphocytes (CD4TLs) and CD8TLs, although it was more marked for the latter (see Table 3).

#### 3.2. CD8TL subsets during exercise

Replicating previous findings (Campbell et al., 2009), exercise resulted in a significantly larger mobilisation of CD8TL effector-memory (+207%) and CD45RA+ effector-memory cells (+239%), as compared to central memory (+108%) or naïve CD8TLs (+58%) (paired samples *t*-tests, all  $t_{(13)} > 4.4$ ,  $p < .01$ ; see Figure 1A). Identifying CD8TL subsets on the basis of CD27 and CD28 expression (yielding early, intermediate, and late differentiated cells, see Table 1), showed that phenotypically late-differentiated cells exhibited a larger increase (+265%) as compared to both intermediate (+154%) and early differentiated cells (+67%). Intermediate

differentiated cells, in turn, showed a larger increase than CD8TL with an early differentiated phenotype (paired samples  $t$ -tests, all  $t_{(13)} > 5.0$ ,  $p < .01$ ; see Figure 1C).

### 3.3. CD8TL subsets post-exercise

Figure 1B and 1D show that the pattern of CD8TL mobilisation seen during exercise was broadly mirrored with the egress 60 minutes post-exercise. Specifically, CD45RA<sup>+</sup> effector memory cells showed a significantly larger decrease (−60%) than effector memory cells (−38%), which both showed a larger decrease than central memory (−30%) and naïve CD8TLs (−29%) (paired samples  $t$ -tests, all  $t_{(13)} > 2.4$ ,  $p < .03$ ). Identifying CD8TL subsets on the basis of CD27 and CD28 expression (see Table 1) revealed that late differentiated cells had the greatest fall in numbers (−60%) compared to cells with an intermediate (−52%) or early (−28%) phenotype (paired samples  $t$ -tests, all  $t_{(13)} > 4.7$ ,  $p < .001$ ; see Figure 1D).

### 3.4. Control trial

Participants who took part in the control trial ( $n = 7$ , condition-order counterbalanced) did not exhibit any significant changes in CD8TL subpopulations or any other leukocyte subset (all pair-wise comparisons  $p > .10$ ).

### 3.5. CMV serostatus and lymphocyte responses to exercise

Repeated measures ANOVA yielded a significant time (Pre, Ex60, Post15, Post60) by serostatus interaction ( $F_{(3, 33)} = 6.3$ ,  $p < .05$ ,  $\eta^2 = 0.37$ , adjusted for age) for CD8TL responses (Figure 2A). Post-hoc analyses revealed that this effect was largely driven by a ~2-fold larger increase in CD8TLs during exercise ( $F_{(1, 11)} = 5.3$ ,  $p < .05$ ,  $\eta^2 = 0.33$ ), as well as a ~2-fold larger

post-exercise egress ( $F_{(1, 11)} = 5.2, p < .05, \eta^2 = 0.32$ ) in CMV<sup>+</sup> individuals (Figure 2C and Figure 2D; all analyses adjusted for age). Comparable results were obtained when using non-parametric statistical tests (Wilcoxon signed-rank test). As shown in Table 4, CMV serostatus was unrelated to age, BMI, leisure time physical activity, fitness, maximum heart rate, or any of the measures of exercise performance. Adjustment for these parameters statistically did not attenuate the observed difference in CD8TL mobilisation. CMV serostatus was unrelated to CD4TL responses (Figure 2B) or total lymphocyte responses (data not shown) (serostatus by time interactions for CD4TL and lymphocytes respectively;  $F_{(3, 33)} = 0.4, p > .7$  and  $F_{(3, 33)} = 1.8, p > .17$ , analyses adjusted for age).

### 3.6. CMV serostatus and CD8TL subset responses to exercise

The differential CD8TL response described above was anticipated because positive CMV serostatus is associated with higher numbers of exercise-responsive late-differentiated/effector-memory subsets (van Lier et al., 2003). This observation was replicated in the current study: compared to CMV<sup>-</sup> individuals, those who were CMV<sup>+</sup> had a ~6-fold greater number of CD45RA<sup>+</sup> effector memory ( $t_{(12)} = -3.3, p < .01$ ) and a ~6-fold greater number of late differentiated CD8TLs ( $t_{(12)} = -3.4, p < .01$ ; independent samples  $t$ -tests).

Figure 3 displays the response of individual CD8TL subpopulations, as identified on the basis of CD45RA and CD27 expression (see Table 1). Repeated measures ANOVA confirmed that serostatus was associated with a larger increase in effector memory cells (CD45RA<sup>-</sup>CD27<sup>-</sup>) (serostatus by time interaction;  $F_{(1, 11)} = 5.3, p < .05, \eta^2 = 0.33$ ) and CD45RA<sup>+</sup> effector memory cells (CD45RA<sup>+</sup>CD27<sup>-</sup>) (serostatus by time interaction;  $F_{(1, 11)} = 15.2, p < .01, \eta^2 = 0.58$ ) (all analyses adjusted for age). The magnitude of post-exercise lymphocytopenia of these subsets

was similarly moderated by CMV serostatus (serostatus by time interactions: for effector memory cells;  $F_{(1, 11)} = 8.5, p < .05, \eta^2 = 0.43$ ; for CD45RA<sup>+</sup> effector memory cells;  $F_{(1, 11)} = 8.5, p < .05, \eta^2 = 0.43$ , both analyses adjusted for age). CMV serostatus was not associated with differential mobilisation or egress of naïve or central memory cells (serostatus by time interactions  $F_{(1, 11)} < 3.5, p > .2$ , adjusted for age).

To test if the larger memory CD8TL responses in CMV<sup>+</sup> individuals were explained by higher basal numbers of these cells, the above analyses were repeated with statistical adjustment for initial values (i.e., entering baseline cell count as a covariate in ANOVAs). After this correction, the interaction effects for effector memory and CD45RA<sup>+</sup> effector memory cells during exercise disappeared ( $F_{(1, 10)} < 4.5, p > .06$ ). Similarly, the augmented post-exercise decrease in CD45RA<sup>+</sup> effector memory cells was no longer statistically different between CMV<sup>+</sup> and CMV<sup>-</sup> individuals after adjusting for baseline cell count ( $F_{(1, 10)} = 3.0, p = .11$ , adjusted for age and initial values), although the larger post-exercise decrease in effector memory cells seen for CMV<sup>+</sup> individuals remained significant after adjustment ( $F_{(1, 10)} = 6.2, p < .05, \eta^2 = 0.38$ , adjusted for age and initial values).

Shown in Figure 4, a comparable pattern of results was found when memory subsets were identified on the basis on CD27 and CD28 expression (see Table 1), demonstrating a significantly larger mobilisation of late differentiated cells in CMV<sup>+</sup> individuals (serostatus by time interaction;  $F_{(1, 11)} = 6.9, p < .05, \eta^2 = 0.39$ , adjusted for age), which was still apparent after controlling for baseline cell count ( $F_{(1, 10)} = 5.5, p < .05, \eta^2 = 0.355$ ), although the post-exercise egress did not reach statistical significance (serostatus by time interaction;  $F_{(1, 11)} = 2.7, p = .13, \eta^2 = 0.20$ , adjusted for age). CMV serostatus did not significantly moderate the responses of

281 intermediate or early differentiated cells (serostatus by time interactions;  $F_{(1, 11)} < 3.8, p > .08$ ; all  
282 analyses adjusted for age).

283



#### 4. Discussion

Recent studies have demonstrated a high specificity in the mobilisation of CD8TL subsets during exercise, whereby a subpopulation of memory cells, i.e., those with strong tissue-migrating capacity and effector potential, preferentially increase in the peripheral blood (Campbell et al., 2009; Simpson et al., 2008; Simpson et al., 2007a). In line with the assumption that this mobilisation is part of an immune surveillance response, we wondered if these memory cells might also show preferential egress from peripheral blood in the post-exercise period. Our results confirmed this expectation, showing a –60% decrease in late differentiated/effector-memory cells, compared to a –25% and –29% fall in total lymphocytes and naïve CD8TLs respectively. Thus, with exercise, cells that are relevant to immune surveillance, i.e., those with proven capacity to initiate immediate effector responses such as target killing, are preferentially redeployed into and out of peripheral blood. As their action is most likely to be required in tissue rather than peripheral blood, it seems probable that the egress, rather than peripheral release, is the most significant aspect of this evolved response.

While the mechanisms responsible for exercise-induced CD8TL mobilisation are well-researched, less is known about the post-exercise egress. Some researchers have contended that the fall in cell number is caused by exercise-induced apoptosis (Mars et al., 1998; Mooren et al., 2002; Mooren et al., 2004). However, both animal and human evidence provide only limited support for this idea (Dhabhar et al., 1995, 1996). For example, although following exercise lymphocyte numbers fall to less than 50% of baseline levels (Hansen et al., 1991; Shephard and Shek, 1999), only <10% of lymphocytes undergo post-exercise apoptosis (Mars et al., 1998; Mooren et al., 2002; Mooren et al., 2004). Moreover, exercise-induced lymphocytopenia has been observed without evidence for apoptosis (Simpson et al., 2007b; Tanimura et al., 2008).

More support exists for the idea that the post-exercise fall in cell numbers is due to lymphocyte homing into peripheral tissues. This migration is postulated to be part of an immune surveillance response (Dhabhar, 2000, 2002; Krüger et al., 2008; Krüger and Mooren, 2007). Cortisol has been proposed as a neuro-endocrine mediator of this lymphocytopenia (Fauci, 1976; McCarthy and Dale, 1988; Onsrud and Thorsby, 1981). However, recent evidence indicates that this is unlikely to explain enhanced egress of late differentiated cells as seen in our study: Dimitrov and co-workers (2009) showed that although cortisol infusion caused lymphocytopenia, this response was restricted to naïve and lymphoid-homing (CD62L<sup>+</sup>) CD8TLs, and that differentiated effector-memory cells were unaffected. It is possible that the subsequent egress may reflect delayed effects of adrenergic stimulation (Ali et al., 2007), which would be consistent with the observation that this delayed decrease so closely parallels the pattern of catecholamine dependent mobilisation.

The CD8TL memory subsets that showed the strongest responses to exercise are known to be selectively increased by latent CMV infection (Appay et al., 2002; van Lier et al., 2003). Significantly, these differentiated memory CD8TL subsets are also known to express higher levels of the  $\beta$ 2-adrenergic receptor, possibly explaining their higher sensitivity to exercise (Dimitrov et al., 2010; Holmes et al., 2005; Karaszewski et al., 1991). Based on these data, we anticipated that CMV serostatus would moderate CD8TL responses to exercise. The results indeed showed that both the mobilisation and post-exercise egress was nearly twice as large in CMV<sup>+</sup> individuals, as compared to CMV<sup>-</sup> individuals. Further analyses confirmed that this augmentation was largely explained by the higher numbers of differentiated memory cells in CMV<sup>+</sup> individuals. For example, the number of CD45RA<sup>+</sup>CD27<sup>-</sup> CD8TLs was ~6-fold higher in CMV<sup>+</sup> participants, which is comparable with what has been reported in the literature

(Chidrawar et al., 2009; Khan et al., 2002; Weinberger et al., 2007). Post-hoc analyses excluded that exercise intensity explained the response differences, as none of the performance indices were related to serostatus (i.e.,  $\dot{V}O_2$ , energy expenditure, heart rate, and RPE). Further analyses also showed that CMV serostatus was unrelated to age, BMI, leisure time physical activity, fitness, and maximum heart rate. To our knowledge, this is the first study to demonstrate that infection history is a determinant of immunological responses to exercise.

CD4TL responses to exercise were not moderated by CMV serostatus. This was not unexpected as CD4TLs and their memory subsets are, compared to CD8TLs, generally less responsive to exercise (Campbell et al., 2009), and the effects of CMV infection on the composition of the CD4TL memory pool are modest (Fletcher et al., 2005; Khan et al., 2002; Pourgheysari et al., 2007; Wikby et al., 2002). Although no further lymphocyte subsets were examined, the null-finding for CD4TLs as well as for total lymphocytes suggests that the observed effects of serostatus are selective for CD8TLs.

What are possible implications of the current findings? Considering that the late differentiated CD8TLs which egress from peripheral blood have a strong tissue homing and inflammatory capacity, it could be argued that amplification of this response may promote protective immune surveillance, and thereby improve clearance of infection and vaccine responses (Edwards et al., 2007; Edwards et al., 2006). Conversely, it might be argued that enhanced mobilisation and tissue migration of CD8TLs may have harmful effects by aggravating inflammatory processes (Dhabhar, 2000, 2002). This may, for example, be relevant in atherosclerosis whereby IFN- $\gamma$  producing CD8TLs accumulate in atherosclerotic plaques (de Boer et al., 2003; de Boer et al., 1999; Gewaltig et al., 2008). Interestingly, CMV also facilitates transmigration of memory CD8TLs via infection of endothelial cells (Burns et al., 1999; Craigen

et al., 1997), and the exaggerated CD8TL mobilisation in CMV<sup>+</sup> individuals might thus further promote cardiovascular disease risk (Strandberg et al., 2003). Moreover, as most cardiovascular diseases are associated with enhanced sympathetic activity (Fisher et al., 2009), it is conceivable that the cardio-protective effects of  $\beta$ -antagonists may be stronger in CMV<sup>+</sup> individuals via blunting of inflammatory CD8TL recruitment (Kuhlwein et al., 2001; Mills et al., 1999).

Another potentially significant implication is that the current observations identify CMV serostatus as a possible mediator or confounder in exercise and behavioural immunology. For example, increased infection rates among athletes following endurance exercise events (the so-called ‘open window hypothesis’) have in part, been attributed to decreased numbers and functional capacity of peripheral blood cells post-exercise (Nieman, 1995; Pedersen and Hoffman-Goetz, 2000). It now seems likely that individual differences in the magnitude of lymphocytopenia, and possibly alterations in cell function post-exercise, are at least in part related to infection history. The same would apply to research linking exercise- (and possibly stress-) induced lymphocyte redeployment to health status (e.g., hypertension) or demographics (e.g., age) (Ceddia et al., 1999; Mazzeo et al., 1998; Mills et al., 2003; Shinkai et al., 1998). This caveat seems to be strengthened by the association of positive CMV serostatus with ethnicity, increased age, lower socio-economic status (Dowd and Aiello, 2009; Lubeck et al., 2010; Staras et al., 2006), as well as health outcomes such as atherosclerotic risk and susceptibility to infection (Dumortier et al., 2008; Hadrup et al., 2006; Olsson et al., 2000; Streblow et al., 2008; Strindhall et al., 2007). Taking a broader perspective, the fact that CMV infection expands a CD8TL population which expresses higher levels of the  $\beta$ 2-adrenergic receptor (Dimitrov et al., 2009; Holmes et al., 2005; Karaszewski et al., 1991) has general relevance to neuro-endocrine immunology. For example, differentiated CD8TLs exhibit unique immunological features, such

as increased IFN- $\gamma$  production and cytotoxicity (Hamann et al., 1997; Sallusto et al., 1999). These functional characteristics may likewise become differentially affected by exercise, and possibly stress, in CMV<sup>+</sup> individuals.

Regarding the limitations of the present study, an important target for future research would be to determine if the enhanced CD8TL response to exercise is attributable to mobilisation of CMV-specific CD8TLs only, or that effector-memory CD8TLs with other antigen-specificities are similarly mobilised. Antigen-specific T lymphocytes can be identified using tetramers, which are synthetic HLA-molecule analogues tagged with an antigen-peptide, that are capable of binding virus-specific T cell receptors. To be able to answer this question conclusively, one would need to use tetramers for all immunogenic CMV epitopes and for all HLA types; we are not aware of a single laboratory that has access to such tetramer diversity. A second limitation of this study is that it is uncertain to what extent our observations would generalise to other latent micro-organisms. A number of viruses are known to affect composition of the lymphocyte pool, but these effects are different from those of CMV. For example, it seems unlikely EBV infection would have the same effect as CMV, because EBV predominantly increases the number of (exercise unresponsive) central-memory cells (Appay et al., 2008; van Lier et al., 2003). Similar to CMV, HIV is known to increase the number of effector-memory cells (Appay et al., 2008; van Lier et al., 2003), which suggests that this infection may also alter CD8TL responses to exercise. A possible third limitation is that participants were all physically fit. Unfit individuals have been shown to exhibit larger CD8TL responses to exercise compared those who are fit (Hong et al., 2004; Hong et al., 2005; Mills et al., 2006), and thus it is conceivable that the effects of CMV serostatus may be weakened or amplified by fitness.

398           In conclusion, the results of the present study have shown that CD8TL mobilisation and  
399 egress in response to intensive exercise are both driven by differentiated effector-memory cells.  
400 Additionally, we have shown, for the first time, that infection history can substantially modulate  
401 this bimodal CD8TL response to exercise by altering the composition of the memory pool.

402

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406

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## Figure legends

### Figure 1

Percentage change ( $\Delta\%$ ) in cell numbers of CD8TLs sub-populations, during exercise (Ex60; A and C), and at 1-hour post-exercise (Post60; B and D). Panel A and B show subsets identified by CD45RA and CD27 expression. Panel C and D show subsets identified by CD27 and CD28 expression. Naïve CD45RA<sup>+</sup>CD27<sup>+</sup>. Central memory (CM) CD45RA<sup>+</sup>CD27<sup>-</sup>. Effector memory (EM) CD45RA<sup>-</sup>CD27<sup>-</sup>. CD45RA<sup>+</sup> Effector memory (RAEM) CD45RA<sup>+</sup>CD27<sup>-</sup>. Early CD27<sup>+</sup>CD28<sup>-</sup>. Inter CD27<sup>+</sup>CD28<sup>-</sup>. Late CD27<sup>-</sup>CD28<sup>-</sup>. <sup>a</sup>  $p < .001$  compared to naïve and CM. <sup>b</sup>  $p < .01$  compared naïve. <sup>c</sup> compared to EM. <sup>d</sup> compared to naïve,  $p < .03$ . <sup>e</sup> compared to CM  $p < .01$ . <sup>f</sup> compared to early  $p < .001$ . <sup>g</sup> compared to inter  $p < .01$  (paired samples  $t$ -tests).

### Figure 2

Changes in A) CD8TLs (cells/ $\mu$ l). B) CD4TLs (cells/ $\mu$ l). C) Percentage change ( $\Delta\%$ ) in CD8TL and CD4TL numbers during exercise (Ex60) D) Percentage change ( $\Delta\%$ ) in CD8TL and CD4TL numbers 60-minutes post-exercise (Post60). \*\*\*  $p < .001$ ; main effects of time, results of repeated measures ANOVA (comparison with baseline values). \*  $p < .05$ , comparing CMV<sup>-</sup> with CMV<sup>+</sup> (ANOVA)

### Figure 3

Changes in CD8TL sub-populations (cells/ $\mu$ l) identified by CD45RA and CD27 expression. A) Naïve CD8TLs (CD45RA<sup>+</sup>CD27<sup>+</sup>). B) Central memory CD8TLs (CM; CD45RA<sup>+</sup>CD27<sup>-</sup>). C) Effector memory CD8TLs (EM; CD45RA<sup>-</sup>CD27<sup>-</sup>). D) CD45RA<sup>+</sup> effector memory CD8TLs (RAEM; CD45RA<sup>+</sup>CD27<sup>-</sup>). \*\*\*  $p < .001$ ; \*\*  $p < .01$ ; main effects of time, results of repeated

measures ANOVA (comparison with baseline values). *See text for 'time by serostatus' interactions.*

#### **Figure 4**

Changes in CD8TL sub-populations (cells/ $\mu$ l) identified by CD27 and CD28 expression. A) Early CD8TLs (CD27<sup>+</sup>CD28<sup>-</sup>). B) Intermediate CD8TLs (CD27<sup>+</sup>CD28<sup>-</sup>). C) Late CD8TLs (CD27<sup>-</sup>CD28<sup>-</sup>). \*\*\*  $p < .001$ ; \*\*  $p < .01$ ; main effects of time, results of repeated measures ANOVA (comparison with baseline values). *See text for 'time by serostatus' interactions.*

#### **Table legends**

##### **Table 1**

Phenotypic identification and functional properties of CD8TL (CD3<sup>+</sup>CD8<sup>+</sup>) subsets

##### **Table 2**

Physiological demands of the exercise and control trials (mean  $\pm$  SD).

\*\*\*  $p < .001$  Comparing exercise with control trial (independent samples  $t$ -test)

RPE; ratings of perceived exertion

##### **Table 3**

Leukocyte subsets before (Pre), after 60 minutes of exercise (EX60), and 15 minutes (POST15) and 60 minutes (POST60) post exercise (mean  $\pm$  SD).

\*\*\*  $p < .001$ , \*\*  $p < .01$ , \*  $p < .05$ , compared to Pre (paired samples  $t$ -test)

# Age as a covariate

665

666 **Table 4**

667 Characteristics of CMV seropositive and seronegative participants (mean  $\pm$  SD). No significant  
668 group differences were observed ( $p > .10$ , independent samples *t*-test).

669 <sup>a</sup> Leisure Time Physical Activity, as assessed by the international physical activity questionnaire  
670 (IPAQ).

671 <sup>b</sup> Data are medians (min-max), differences assessed using a Mann-Whitney U Test.

672

**Table 1.** Phenotypic identification and functional properties of CD8TL (CD3<sup>+</sup>CD8<sup>+</sup>) subsets

Cell description	Identification	Migration preference	Effector potential	Reference
Naïve	CD45RA <sup>+</sup> CD27 <sup>+</sup>	Lymphoid tissue	–	Hamann et al. 1997; Romero et al 2007
Central memory	CD45RA <sup>+</sup> CD27 <sup>–</sup>	Lymphoid tissue	–	Hamann et al. 1997; Romero et al 2007
Effector memory	CD45RA <sup>–</sup> CD27 <sup>–</sup>	Peripheral tissue	+	Hamann et al. 1997; Romero et al 2007
CD45RA <sup>+</sup> effector memory	CD45RA <sup>+</sup> CD27 <sup>–</sup>	Peripheral tissue	++	Hamann et al. 1997; Romero et al 2007
Early	CD27 <sup>+</sup> CD28 <sup>+</sup>	Lymphoid tissue	–	Appay et al. 2002
Inter	CD27 <sup>+</sup> CD28 <sup>–</sup>	Peripheral tissue	+	Appay et al. 2002
Late	CD27 <sup>–</sup> CD28 <sup>–</sup>	Peripheral tissue	++	Appay et al. 2002



**Table 2.** Physiological demands of the exercise and control trials (mean  $\pm$  SD)

	Exercise	Control
	<i>n</i> = 14	<i>n</i> = 7
$\dot{V}O_2$ (ml·kg <sup>-1</sup> ·min <sup>-1</sup> )	45.6 $\pm$ 3.3***	3.6 $\pm$ 0.5
$\dot{V}O_2$ (% $\dot{V}O_{2max}$ )	78.7 $\pm$ 1.9***	6.4 $\pm$ 1.1
Energy expenditure (kcal)	1011.0 $\pm$ 85.4***	80.4 $\pm$ 13.8
Heart rate (bpm)	162 $\pm$ 13***	51 $\pm$ 7
Heart rate (% max)	88.0 $\pm$ 4.4***	28.9 $\pm$ 3.4
RPE	14 $\pm$ 2***	6 $\pm$ 0

\*\*\*  $p$  < .001 Comparing exercise with control trial (independent samples  $t$ -test)  
RPE; ratings of perceived exertion

**Table 3.** Leukocyte subsets before (Pre), after 60 minutes of exercise (EX60), and 15 minutes (POST15) and 60 minutes (POST60) post exercise (mean  $\pm$  SD)

Cells x 10 <sup>9</sup> /L	Pre	EX60	POST15	POST60	Main effects of time #
Leukocytes	6.29 $\pm$ 1.18	11.66 $\pm$ 3.13***	8.19 $\pm$ 3.01***	7.73 $\pm$ 3.51***	$F_{(3, 33)} = 15.8; p < .001$
Lymphocytes	2.59 $\pm$ 0.59	5.51 $\pm$ 1.82***	2.94 $\pm$ 1.20	1.93 $\pm$ 0.94***	$F_{(3, 33)} = 150.0; p < .001$
Monocytes	0.53 $\pm$ 0.19	0.89 $\pm$ 0.41	0.54 $\pm$ 0.25	0.51 $\pm$ 0.23	$F_{(3, 33)} = 0.4; p = \text{NS}$
Granulocytes	3.17 $\pm$ 0.92	5.26 $\pm$ 2.10***	4.71 $\pm$ 1.63***	5.29 $\pm$ 2.15**	$F_{(3, 33)} = 16.9; p < .001$
CD4TLs	1.09 $\pm$ 0.36	1.65 $\pm$ 0.81***	1.15 $\pm$ 0.63	0.86 $\pm$ 0.47***	$F_{(3, 33)} = 57.9; p < .001$
CD8TLs	0.63 $\pm$ 0.24	1.33 $\pm$ 0.60***	0.71 $\pm$ 0.29*	0.40 $\pm$ 0.16***	$F_{(3, 33)} = 62.2; p < .001$

\*\*\*  $p < .001$ , \*\*  $p < .01$ , \*  $p < .05$ , compared to Pre (paired samples  $t$ -test)

# Age as a covariate

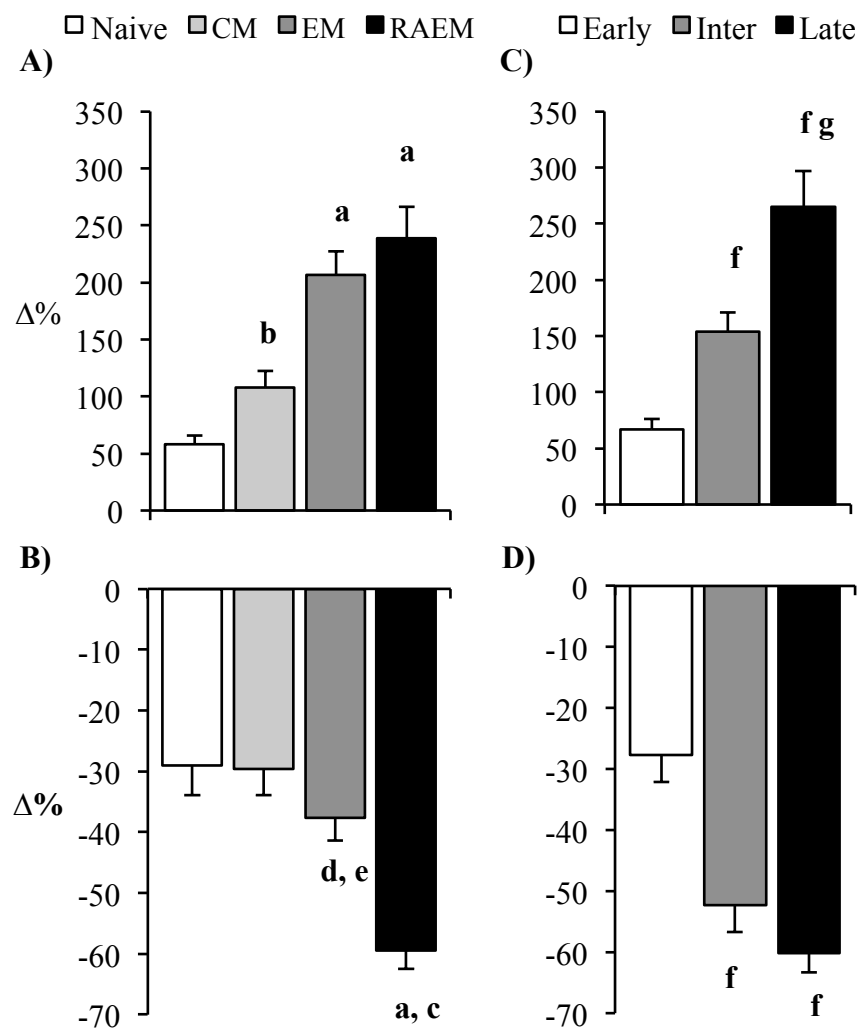
**Table 4.** Characteristics of CMV seropositive and seronegative participants (mean  $\pm$  SD). No significant group differences were observed ( $p > .10$ , independent samples  $t$ -test).

	CMV <sup>+</sup>	CMV <sup>-</sup>
	$n = 7$	$n = 7$
<b>Subject characteristics at baseline</b>		
Age (years)	34.5 $\pm$ 12.0	33.7 $\pm$ 15.9
BMI (kg·m <sup>-2</sup> )	24.1 $\pm$ 3.6	23.3 $\pm$ 1.4
LTPA <sup>a</sup> (Median MET·min·week <sup>-1</sup> ) <sup>b</sup>	3960 (2853-4850)	2880 (1080-7290)
Measured $\dot{V}O_2$ max	58.2 $\pm$ 5.1	57.8 $\pm$ 3.8
Maximum heart rate at $\dot{V}O_2$ max (bpm)	181 $\pm$ 10	188 $\pm$ 9
<b>Exercise trial results</b>		
$\dot{V}O_2$ (ml·kg <sup>-1</sup> ·min <sup>-1</sup> )	46.0 $\pm$ 3.6	45.1 $\pm$ 3.2
Energy expenditure (kcal)	1040.16 $\pm$ 82.44	981.77 $\pm$ 83.80
Heart rate (bpm)	159 $\pm$ 11	166 $\pm$ 14
RPE	13 $\pm$ 1	14 $\pm$ 2

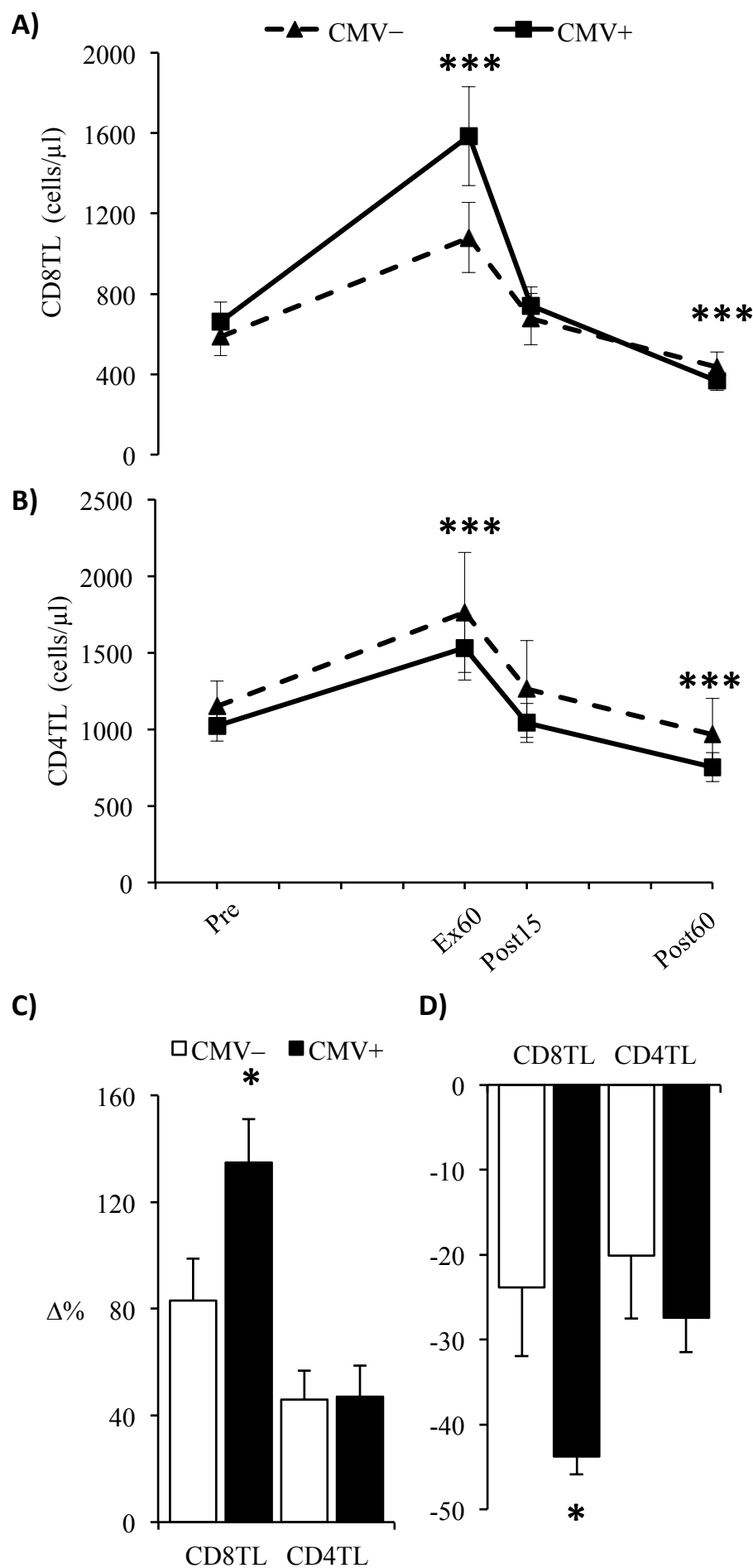
<sup>a</sup> Leisure Time Physical Activity, as assessed by the international physical activity questionnaire (IPAQ).

<sup>b</sup> Data are medians (min-max), differences assessed using a Mann-Whitney U Test.

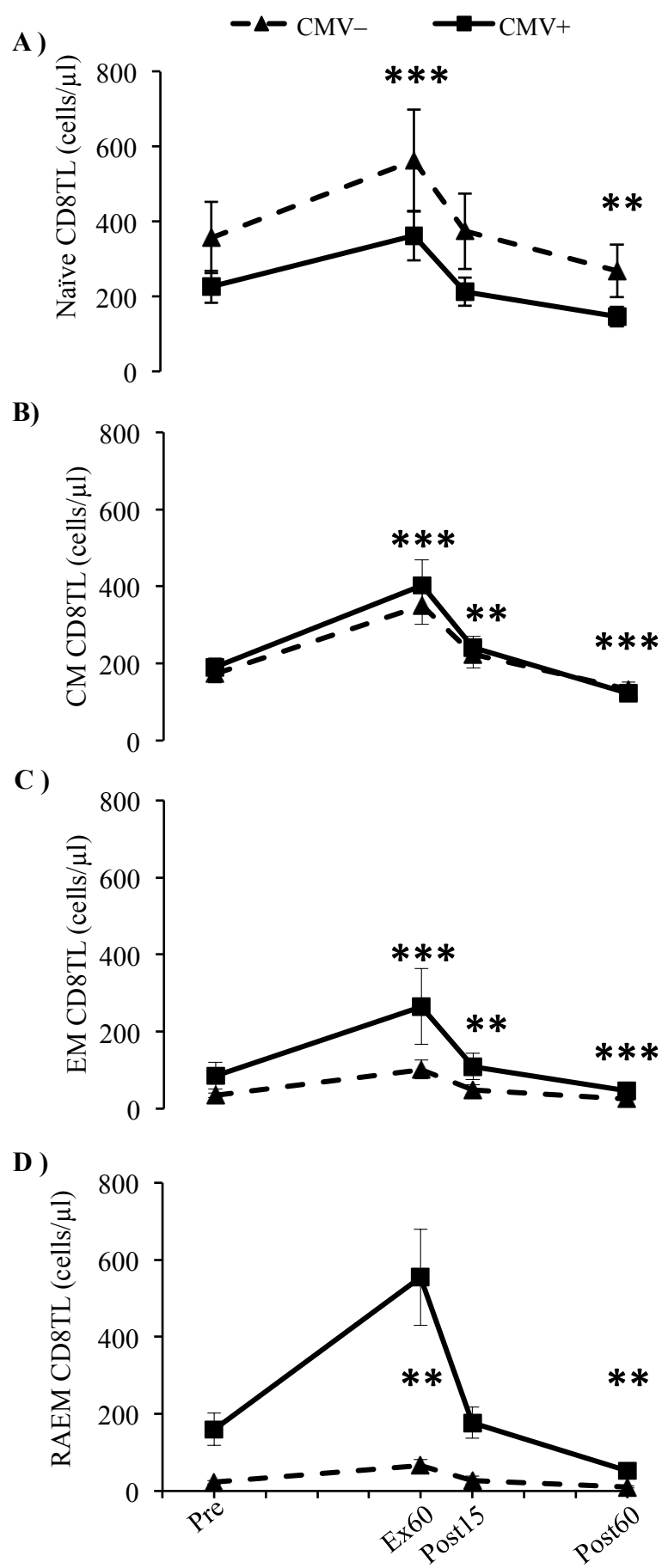
**Figure 1.** Turner. CMV amplifies CD8T cell responses to exercise



**Figure 2.** Turner. CMV amplifies CD8T cell responses to exercise



**Figure 3.** Turner. CMV amplifies CD8T cell responses to exercise



**Figure 4.** Turner. CMV amplifies CD8T cell responses to exercise

